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1 **Short report**

2 **Dietary n-6 to n-3 fatty acid ratio is related to liver fat content independent of genetic effects:**
3 **evidence from the monozygotic co-twin control design**

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16 Keywords: identical twins; twin study; liver fat; nutrition; omega-6: omega-3 ratio

17 **Abstract**

18 Background & aim: Lifestyle changes focusing on diet and exercise remain the cornerstone of the
19 treatment of non-alcoholic fatty liver disease (NAFLD). The present co-twin control study of
20 monozygotic (MZ) twin pairs was designed to identify nutritional factors potentially involved in the
21 pathogenesis of NAFLD.

22 Methods: Cross-sectional study of 50 MZ twin pairs (age range: 23-36 years), of which ten pairs were
23 discordant for liver fat (liver fat percentage of one twin \leq 5% and his/her co-twin $>$ 5% and a
24 difference between co-twins of $>$ 5%) as determined by magnetic resonance spectroscopy. Nutrient
25 intake was calculated from 3-day food records.

26 Results: Among the ten liver fat-discordant twin pairs, the n-6: n-3 ratio was significantly higher in
27 the twins with higher liver as compared to their co-twins with lower liver fat (6.6: 1 vs. 3.2: 1, p -value
28 = 0.005). In multiple regression analysis of within-pair differences including all 50 twin pairs, a
29 higher n-6: n-3 ratio was significantly associated with a higher liver fat percentage within MZ twin
30 pairs after adjustment for body mass index, energy intake and other covariates (standardized beta=
31 0.43, p -value = 0.001).

32 Conclusions: Our findings suggest that the n-6: n-3 ratio is a promising dietary agent for the
33 prevention and treatment of NAFLD. Clinical trials are required to better understand causal
34 relationships and required doses.

35

36 **Introduction**

37 Non-alcoholic fatty liver disease (NAFLD), characterized by fat accumulation in more than 5% of
38 hepatocytes in the absence of excessive alcohol consumption, is the most common chronic liver
39 disease in the world. It affects up to a quarter of the general population, depending on the country
40 studied¹. The disease spectrum includes simple steatosis, steatohepatitis, advanced fibrosis and
41 cirrhosis which can progress to liver failure¹.

42 The heritability of NAFLD is low, underscoring the importance of environmental factors in its
43 etiology². Currently, there are no approved pharmacotherapies for patients with NAFLD. Therefore,
44 weight reduction and lifestyle changes through diet and exercise form the first-line therapy for
45 treatment³. Diets high in saturated fatty acids and refined carbohydrates could exacerbate NAFLD⁴,
46 while Mediterranean dietary patterns might reduce liver fat even in the absence of weight loss⁵.
47 Randomized controlled trials suggest that supplementation with omega-3 (n-3) polyunsaturated fatty
48 acids (PUFAs) has therapeutic potential for treating patients with NAFLD⁶.

49 Another powerful study design that allows stronger inference about causality than observational
50 studies of unrelated individuals is the co-twin-control design of disease-discordant monozygotic
51 (MZ) twin pairs because MZ twins are genetically identical at the DNA sequence level and
52 discordance within pairs must thus be of environmental origin. Our previous twin study suggests that
53 metabolic abnormalities in acquired obesity are tightly linked to liver fat content⁷. The present study
54 of MZ twins was designed to identify nutritional factors potentially involved in the pathogenesis of
55 NAFLD.

56 **Research design and methods**

57 TwinFat study participants were enrolled from two Finnish population-based longitudinal studies of
58 five consecutive birth cohorts of young adult twins⁸. Participants were invited to the TwinFat study
59 based on their reported body mass index (BMI) during these studies (at the age of 23 to 27 years)
60 with the aim to represent a wide range of intra-pair differences in BMI. Participants were then
61 weighed and their height was measured while barefoot and in light clothing by trained research
62 nurses. Whole-body fat was measured by dual energy x-ray absorptiometry, abdominal
63 subcutaneous and intra-abdominal fat by magnetic resonance imaging and liver fat percentage by
64 magnetic resonance spectroscopy as described earlier⁹. The homeostasis model assessment of
65 insulin resistance (HOMA-IR) was calculated based on 2-hour oral glucose tolerance tests. Fasting
66 plasma cholesterol and triglyceride concentrations were determined with enzymatic methods.
67 Supine blood pressure was measured using an automatic digital sphygmomanometer (mean of three
68 measurements).

69 Nutrient intake was calculated from 3-day food records by the Diet32 program (Aivo), based on a
70 national Finnish database (Fineli, www.fineli.fi, National Institute for Health and Welfare, Nutrition
71 Unit, Helsinki, Finland). The sports activity index was calculated from the Baecke questionnaire¹⁰.
72 Out of 107 twin individuals with data on liver fat and food records, one twin individual was excluded
73 because of missing information on physical activity, two twin individuals because of implausible
74 energy intakes (one woman reported < 714 kcal/day, and one men > 4200 kcal/day) and another four
75 twin individuals because of missing co-twin information. Thus, the final analytic sample consisted of
76 50 MZ twin pairs, of which 10 twin pairs were discordant for liver fat. Liver fat discordance was
77 defined as the liver fat percentage of one twin $\leq 5\%$ and his/her co-twin $> 5\%$ and a difference between
78 co-twins of $> 5\%$ resulting in identification of ten such pairs. The study protocol was in accordance
79 with the Helsinki Declaration and approved by local Ethical Committees. Written informed consent
80 was given by all twins.

81 Data were reported as median and interquartile ranges. Differences between discordant twin pairs
82 were analysed using Wilcoxon signed rank test. In all twin pairs, multiple linear regression analysis
83 was used to relate within-pair differences in log transformed liver fat to within-pair differences in the
84 omega-6 (n-6) to n-3 ratio adjusted for covariates. Statistical analyses were performed using
85 Stata version 13 (Stata Corp, College Station, TX).

86 **Results**

87 The descriptive characteristics (clinical, behavioral, nutritional and liver fat) of the 100 twin
88 individuals (mean age 29.9 years (range: 23-36 years) and of the subset of ten liver fat-discordant
89 twin pairs are shown in **Table 1**. Among these ten pairs, the twins with higher liver fat had more body
90 fat and a worse metabolic profile than their co-twins with lower liver fat. The twins with higher and
91 lower liver fat did not differ in physical activity indices and energy intake, or the intake of protein,
92 fat and carbohydrates. In every single discordant pair, the twin with the higher liver fat percentage
93 had consistently a higher n-6: n-3 ratio than his or her co-twin with less liver fat (**Supplementary**
94 **Figure**). The twins with the lower liver fat percentage had a mean n-6: n-3 ratio of 3.2: 1, while the
95 ratio in the co-twins with higher liver fat percentage was 6.6: 1 (p -value = 0.005 for the difference
96 between pairs).

97 The results from the within-pair linear regression on all 50 MZ pairs are presented in **Table 2**. The n-
98 6: n-3 ratio and BMI were significant predictors and of about equal importance in predicting within-
99 pair differences in liver fat. The gender interaction (male vs. female pairs) was not significant.
100 Additional adjustment for intra-abdominal fat (standardized beta= 0.37, p =0.08) did not change the
101 effect estimate for the n-6: n-3 ratio (standardized beta= 0.39, p -value = 0.002), but removed the
102 influence of BMI (standardized beta= 0.13, p -value = 0.52). In a model that additionally adjusted for
103 sucrose, the effect size for sucrose was not significant (standardized beta= -0.12, p -value = 0.28), and
104 the effect estimate for the n-6: n-3 ratio remained unchanged (standardized beta= 0.40, p -value =
105 0.001).

106 **Discussion**

107 To our knowledge, this is the first study that evaluated nutrient intake differences in MZ twin pairs
108 discordant for liver fat. Among the 10 twin pairs discordant for liver fat, the co-twins with higher
109 liver fat reported a higher dietary n-6: n-3 ratio than his or her co-twin in every single pair. We
110 employed the MZ co-twin control design, which is analogue to a case-control study, where the twin
111 with the higher liver fat is the case and the co-twin with the lower liver fat the control, with the
112 additional advantage that cases and controls are naturally matched for several factors including age,
113 sex and genotype.

114 Due to modern agriculture and the popularity of fast foods, Western diets contain excessive amounts
115 of n-6 PUFAs and low amounts of n-3 PUFAs. An overconsumption of n-6 PUFAs coupled with
116 under-consumption of n-3 PUFAs may lead to inflammation and pro-inflammatory cytokine
117 production involved in many disease processes¹¹. In addition, n-3 PUFAs may influence the intestinal
118 microbiota¹² and thereby modulate NAFLD susceptibility. Recent meta-analysis of randomized
119 controlled trials suggest that n-3 PUFA supplementation can reduce liver fat and glucose control in
120 patients with NAFLD, at amounts that can be easily obtained through the habitual diet⁶.

121 In the present study of liver-fat discordant MZ twin pairs, a mean n-6: n-3 ratio of 3.2: 1 was
122 associated with normal liver fat content, while a mean ratio of 6.6: 1 was associated with increased
123 liver fat content independent of genetic and familial confounding factors. Dietary advice for
124 preventing and treating NAFLD should focus on shifting consumption away from processed foods

125 (such as refined vegetable oils rich in n-6 fatty acid used for deep frying) towards fresh foods (such
 126 as vegetable oils used in salads), and by increasing fish intake while decreasing meat intake¹¹. Our
 127 findings could have important implications for the dietary management of NAFLD and encourage
 128 clinical trials to better understand causal relationships and required doses.

129 **Competing interest statement**

130 The authors declare no competing interests.

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- 165

Table 1. Clinical characteristics, physical activity and macronutrient intakes of the monozygotic twins

	All twins	Twin pairs discordant for liver fat (within-pair difference in liver fat > 5%) ¹		p-value ²
		Co-twins with liver fat ≤ 5%	Co-twins with liver fat > 5%	
Demographic factors				
Number (n) of individuals ³	100	10	10	
Males (n)	46	6	6	
Liver fat >5% (n)	22	0	10	
Liver fat (%) ⁴	1.0 (0.5, 3.9)	1.1 (0.8, 3.2)	9.4 (7.3, 9.4)	
Clinical parameters ⁴				
BMI (kg/m ²)	26.6 (23.7, 29.8)	25.7 (22.9, 27.8)	31.8 (27.6, 35.0)	0.005
Body fat (%)	32.4 (26.9, 40.7)	27.7 (21.2, 38.8)	38.3 (31.9, 43.4)	0.007
Intra-abdominal fat (dm ³)	807 (422, 1355)	604 (439, 11165)	1709 (1416, 2417)	0.007
Subcutaneous fat (dm ³)	3727 (2449, 5513)	3263 (2150, 5498)	5768 (3195, 6965)	0.007
LDL-cholesterol (mmol/l)	2.7 (2.3, 3.2)	2.7 (2.5, 2.9)	3.3 (2.7, 3.8)	0.07
HDL-cholesterol (mmol/l)	1.4 (1.2, 1.7)	1.5 (1.3, 1.7)	1.2 (1.1, 1.4)	0.02
Triglycerides (mmol/l)	1.0 (0.7, 1.3)	0.7 (0.5, 1.1)	1.3 (1.0, 2.2)	0.01
HOMA-IR index	1.4 (0.8, 2.0)	1.1 (0.5, 1.8)	2.4 (1.8, 2.8)	0.01
Systolic blood pressure (mmHg)	124 (118, 138)	131 (122, 140)	139 (124, 148)	0.36
Diastolic blood pressure (mmHg)	70 (65, 80)	70 (70, 78)	79 (70, 80)	0.18
Physical activity ⁴				
Sports activity index	2.8 (2.3, 3.5)	3.3 (2.3, 4)	2.4 (1.8, 3.5)	0.33
Leisure time activity index	2.8 (2.5, 3.3)	2.8 (2.5, 3.0)	2.9 (2.5, 3.3)	>0.99
Work activity index	2.5 (2.0, 3.3)	2.3 (2.1, 3.0)	2.6 (1.9, 2.9)	0.14
Total energy intake (kcal) ⁴	2075 (1774, 2551)	2169 (1809, 2382)	2611 (2319, 2800)	0.07
Macronutrient intake (% energy intake) ⁴				
Carbohydrates	43.1 (38.3, 48.1)	43.5 (37.9, 50.0)	37.4 (33.4, 44.0)	0.09
Protein	16.5 (14.8, 18.8)	18.0 (14.2, 22.9)	16.0 (14.1, 18.9)	0.24
Total fat	35.2 (30.6, 39.0)	32.4 (26.3, 38.9)	39.0 (31.6, 42.8)	0.11
Sucrose	9.7 (6.0, 13.0)	4.8 (3.8, 7.0)	8.8 (7.9, 11.2)	0.04
Saturated fatty acids	13.5 (11.1, 16.2)	11.3 (10.9, 13.8)	13.1 (10.7, 16.3)	0.58
Monounsaturated fatty acids	10.6 (9.0, 12.3)	8.8 (7.4, 12.8)	12.0 (9.6, 13.4)	0.24
Polyunsaturated fatty acids	4.5 (3.8, 5.8)	4.1 (3.5 5.5)	5.6 (4.8, 5.8)	0.09
Omega-6/omega-3 ratio ⁴	4.8 (3.0, 6.1)	3.2 (2.3, 5.4)	6.6 (3.8, 7.4)	0.005

Abbreviations: BMI, body mass index; LDL, low-density lipoprotein; HDL, high -density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance

¹Discordance was defined as the liver fat percentage of one twin ≤ 5% and the liver fat percentage of his/her co-twin > 5% and a within-pair difference in liver fat of > 5%. The difference in liver fat percentage between discordant co-twins ranged from 5 to 20%.

²P-values are from Wilcoxon signed rank test comparing co-twins discordant for liver fat.

³The 100 monozygotic twin individuals are from 50 complete twin pairs of which 10 twin pairs are discordant for liver fat.

⁴Data are median and interquartile ranges.

Table 2. Multiple linear regression analysis predicting (log transformed) liver fat within 50 pairs of monozygotic twins

Dependent variable: Within-pair difference (Δ) in log liver fat	Unstandardized beta coefficient	Standard error	Standardized beta coefficient	p-value
Sex (Female twin pairs)	-0.57	0.23	-0.31	0.017
Age of the twin pairs (years)	-0.02	0.02	-0.09	0.42
Δ Omega-6/omega-3 ratio	0.24	0.06	0.43	0.001
Δ Total energy intake (per 100 kcal)	0.00	0.02	-0.02	0.83
Δ Body mass index (kg/m^2)	0.10	0.03	0.42	0.003
Δ Alcohol intake (% energy intake)	-0.02	0.02	-0.12	0.30
Δ Sports activity index	-0.14	0.10	-0.17	0.17
$R^2=0.49$				

Supplementary Figure. Omega-6/omega-3 ratio in ten monozygotic twin pairs discordant for liver fat

